Morphology and possible mycobiont (*Suillus pictus*) of a tuberculate ectomycorrhiza on *Pinus strobus*[1,2]

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Received August 14, 1985

RANDALL, B. L., and L. F. GRAND. 1986. Morphology and possible mycobiont (*Suillus pictus*) of a tuberculate ectomycorrhiza on *Pinus strobus*. Can. J. Bot. **64**: 2182–2191.

Suillus pictus was consistently associated with tuberculate ectomycorrhizae (tubercles) on *Pinus strobus* in North Carolina. Histological, cultural, and chemical reaction studies were used to examine this association. Tubercles were examined by light and scanning electron microscopy. Clamp connections, a reduced to absent tannin layer, and appressed mantle hyphae were observed. Naturally occurring rhizomorphs of *Suillus pictus* and tubercles have a similar morphology consisting of an outer pseudoparenchymatous sheath, large vessel-like cells, and a prosenchymatous layer between the sheath and the vessel-like cells. Similar macroscopic chemical color reactions to 10% $FeSO_4$, 15% KOH, concentrated NH_4OH, concentrated H_2SO_4, and Melzer's reagents were observed on rhizomorphs of *S. pictus* and tubercles, surface of tubercles, and stipes of *S. pictus*. Chemical color reactions and growth characteristics of *S. pictus* and the tubercle mycobiont were similar on different agar media.

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Le *Suillus pictus* est régulièrement associés à des ectomycorhizes tuberculées (tubercules) chez le *Pinus strobus* en Caroline du Nord. Cette association a été étudiée par des méthodes histologiques et culturales ainsi qu'à l'aide de réactions chimiques. Les tubercules ont été examinés par microscopie photonique et par microscopie électronique en balayage. On y retrouve des boucles d'anastomose, une couche tannifère réduite ou absente et des hyphes du manteau apprimés. Les rhizomorphes trouvés en mature ont une morphologie similaire qui comporte une zone externe pseudoparenchymatique, de grandes cellules en forme de vaisseaux au centre et une couche prosenchymatique entre la zone externe et les cellules en forme de vaisseaux. Des réactions chimiques similaires sont obtenues lorsque du $FeSO_4$ à 10%, du KOH à 15%, du NH_4OH concentré, du H_2SO_4 concentré et du réactif de Melzer sont appliqués sur les rhizomorphes du *S. pictus*, sur les tissus des tubercules, sur la surface des tubercules et sur les pieds de fructifications du *S. pictus*. Les réactions macrochimiques et les caractéristiques générales du *S. pictus* demeurent les mêmes sur différents milieux de culture.

[Traduit par la revue]

Introduction

Tuberculate forms of ectomycorrhizae have been found on species of *Pinus, Tsuga, Pseudotsuga, Quercus* (Trappe 1965), and *Photinia* (Grand 1971). Species of *Suillus* and *Rhizopogon* have been suggested or identified as the mycobionts of a number of tuberculate ectomycorrhizae (tubercles) on conifers. Zak (1971) demonstrated that *Rhizopogon vinicolor* A. H. Sm. is the mycobiont of a black tubercle on *Pseudotsuga menziesii* (Mirb.) Franco var. *menziesii* and var. *glauca* (Beissn.) Franco. The mycobiont of a yellow tubercle on *Tsuga mertensiana* (Bong.) Carr, and species of *Abies* and *Pinus* in the Pacific Northwest, is *Rhizopogon cokeri* A. H. Sm. (J. M. Trappe, personal communication to L. F. Grand). *Rhizopogon parasiticus* Coker & Totten has been suggested as the mycobiont of a buff-colored tubercle on *Pinus taeda* L. and *P. echinata* Miller (Totten 1923). However, *R. parasiticus* was also suggested as a parasite of the tubercle and not the actual mycobiont (Smith and Zeller 1966).

Melin reported tubercles formed by associations between *Boletus luteus* Fr. (= *Suillus luteus* (L. : Fr.) S. F. Gray) and *Pinus sylvestris* L., "*Mycelium Radicis sylvestris* α," and *P. sylvestris* (Melin 1922), and *Boletus variegatus* Sow. : Fr. (= *Suillus variegatus* (Sow. : Fr.) Kuntze) and *Pinus montana* Miller (Melin 1924). Chumak (1981) described tubercles formed by *Suillus* spp. and *P. sylvestris* in the USSR. A tuberculate ectomycorrhiza was reported on *Pinus strobus* L. in the United States by McDougal (1922). Dominik and Ferchau (1958) examined the morphology of this tubercle from the United States and noted an absence of tubercles in *P. strobus* plantations in France and Poland. Doak (1934) reported pure culture synthesis of ectomycorrhizae by *S. pictus* (Peck) A. H. Sm. & Thiers (= *Bolentinus pictus* (Peck) Peck) with *P. strobus* but did not indicate the formation of a tuberculate type.

A consistent association between the occurrence of basidiocarps of *Suillus pictus* and tubercles was observed in plantations and natural populations of *P. strobus* in the mountains and Piedmont of North Carolina (B. L. Randall, unpublished data). *Suillus americanus* (Peck) Snell : Slipp & Snell and *S. granulatus* (L. : Fr.) Kuntze also were consistently associated with *P. strobus* but not as consistently with tubercles.

The possibility that *S. pictus*, a commonly associated mycobiont with *P. strobus* (Smith and Thiers 1971; Grand 1970; Snell and Dick 1970), is the mycobiont of the tubercle prompted further examination. The objectives of this study were (*i*) to describe the morphology of tubercles by light and scanning electron microscopy and (*ii*) to evaluate the hypothesis that *S. pictus* is the mycobiont of the tuberculate ectomycorrhiza on *P. strobus*.

[1]Paper No. 10037 of the Journal Series of the North Carolina Agricultural Research Service, Raleigh, NC, U.S.A. 27695-7601.

[2]The use of trade names in this publication does not imply endorsement by the North Carolina Agricultural Research Service of the products named nor criticism of similar ones not mentioned.

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Printed in Canada / Imprimé au Canada

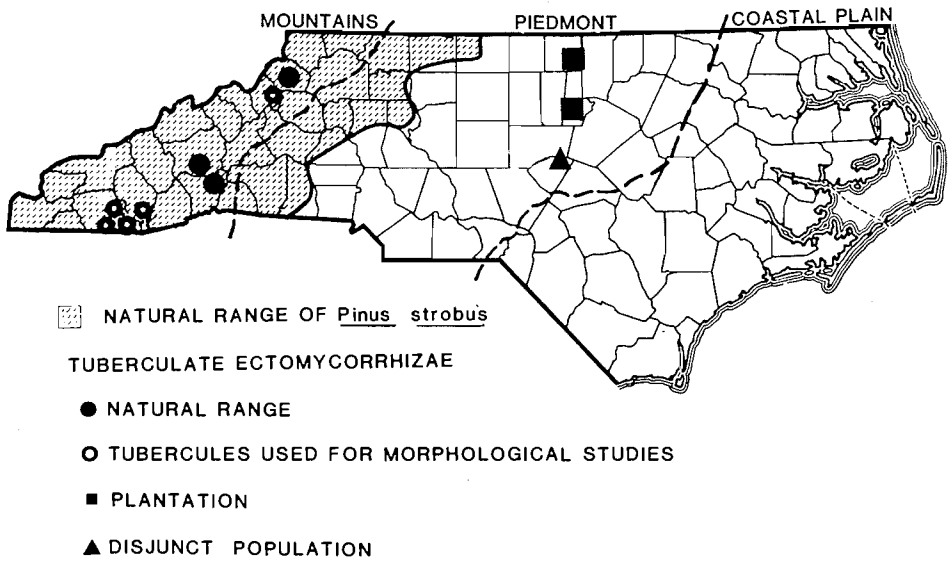

FIG. 1. Natural range of *Pinus strobus* and distribution of tuberculate ectomycorrhizae in North Carolina.

Materials and methods

Tubercles

Tubercles from five mountain populations (Macon, Jackson, and Avery counties) and a disjunct Piedmont population (Chatham County) of *Pinus strobus* in North Carolina were selected for study (Fig. 1). Tubercles were separated by hand from the duff and soil in the field and stored in plastic bags on ice for transport to the laboratory. Macroscopic morphological features and chemical color reactions were noted on fresh samples at 7–40×. Other samples were stored at 4°C prior to processing.

Tubercles selected for histological examination were fixed in Formalin – acetic acid – alcohol (FAA), dehydrated in a tertiary butyl alcohol series (Johansen 1940), and infiltrated and embedded in TissuePrep® (Fisher Scientific Company, Chemical Manufacturing Division, Fair Lawn, NJ, U.S.A. 07410). Embedded tissue was sectioned at 8–12 μm on a rotary microtome, mounted on glass slides with Haupt's adhesive, and stained with either a modified Conant's quadruple stain (Johansen 1940) or safranin and fast green (Marx and Davey 1969).

Fresh tubercles selected for scanning electron microscopy (SEM) were fixed in FAA, placed in Cryoform® (International Equipment Company, Needham Heights, MA, U.S.A. 02194), sectioned at 20–35 μm on a freezing microtome, dehydrated first in ethanol, then in a graded ethanol–freon series (Anderson 1951), and critical point dried in Freon 13® (E. I. DuPont de Nemours & Company, Wilmington, DE, U.S.A. 19898). Dried sections were mounted on metal stubs, coated with gold, and observed and photographed with an ETEC Autoscan electron microscope.

Rhizomorphs

Rhizomorphs attached to stipes of basidiocarps of *S. pictus* and those attached to tubercles found in the soil beneath basidiocarps of *S. pictus* were fixed in FAA and prepared for sectioning as previously described.

Chemical reagent color reactions

Tubercles were sectioned by hand with a razor blade and placed in wells of a porcelain spot plate. Five chemical reagents were tested individually on sections by placing two or three drops of each reagent on one or two sections. Color reactions were observed at 7–40×. Chemical reagents used were Melzer's solution (Singer 1975), aqueous 10% $FeSO_4$, 15% aqueous KOH, concentrated NH_4OH, and concentrated H_2SO_4 (Zak 1973). Fumes of NH_3 were obtained by placing an eyedropper in a bottle containing concentrated NH_4OH, taking up the solution, and then releasing it. The small amount adhering on the inner wall of the eyedropper quickly volatilized to NH_3. Squeezing the bulb of the eyedropper released NH_3 fumes onto the tissue being tested.

Sections of rhizomorphs from both tubercles and *S. pictus* basidiocarps were tested with the same five reagents. Basidiocarps of *S. pictus* were tested; however, a weaker concentration of KOH (10%) was used.

Capitalized color terms used for color reactions are from Ridgeway (1912).

Cultural characteristics

Cultures were obtained from basidiocarps of *S. pictus* and from tubercles. Basidiocarps were aseptically split in half, and tissue was removed from the pileus and stipe flesh and plated on modified Melin–Norkrans (MMN) medium (Marx 1969). Tubercles were surface disinfected for 30–60 s in 30% H_2O_2, sliced into sections, and plated on MMN medium. Isolates were obtained from tubercles harvested through the year or from tubercles held in storage at 4°C for up to 3 months. Isolates of *S. pictus* (SP2, SP7) and tubercles (T50, T100, T101, T102, T104) were grown on MMN, modified Hagems agar (MH) (Modess 1941), and potato dextrose agar (PDA) media at room temperature (20–24°C) for 20–25 days. Colors of mycelial mats (surface and reverse) and colors of the medium adjacent to and beneath mycelial mats were noted. Colony topography and morphology were also noted. Three plate cultures of each isolate on each medium were tested for color reactions to 10% aqueous NH_4OH and Melzer's solution.

Hyphal mounts for examination by light microscopy were prepared by placing plugs of mycelium grown on MMN medium onto sterile glass slides supported on glass rods in sterile glass Petri dishes. Sterile water was added to filter paper in the bottom of the Petri plates to make moist chambers. Sterile cover slips were placed on top of the plugs. Moist chambers were kept at room temperature for 4–7 days. Cover slips were then removed and placed on a drop of aniline blue – lactophenol on a glass slide. Hyphae were observed and photographed with a Leitz Ortholux phase microscope (100× and 400×).

Growth-rate study

Tuberculate isolates (T3, T50, T100, T102, and T104) were used for diameter growth-rate studies. Initial cultures were grown on MMN medium for 12 days. Plugs (5 mm diameter) cut from the growing edge of a colony were plated (one plug per plate) on 25 mL MMN medium in each of thirty 100 mm diameter Petri plates. Five plates of each isolate were incubated at each of six temperatures: 12, 16, 20, 24, 28, and 32°C. Colony diameter was measured at 3- to

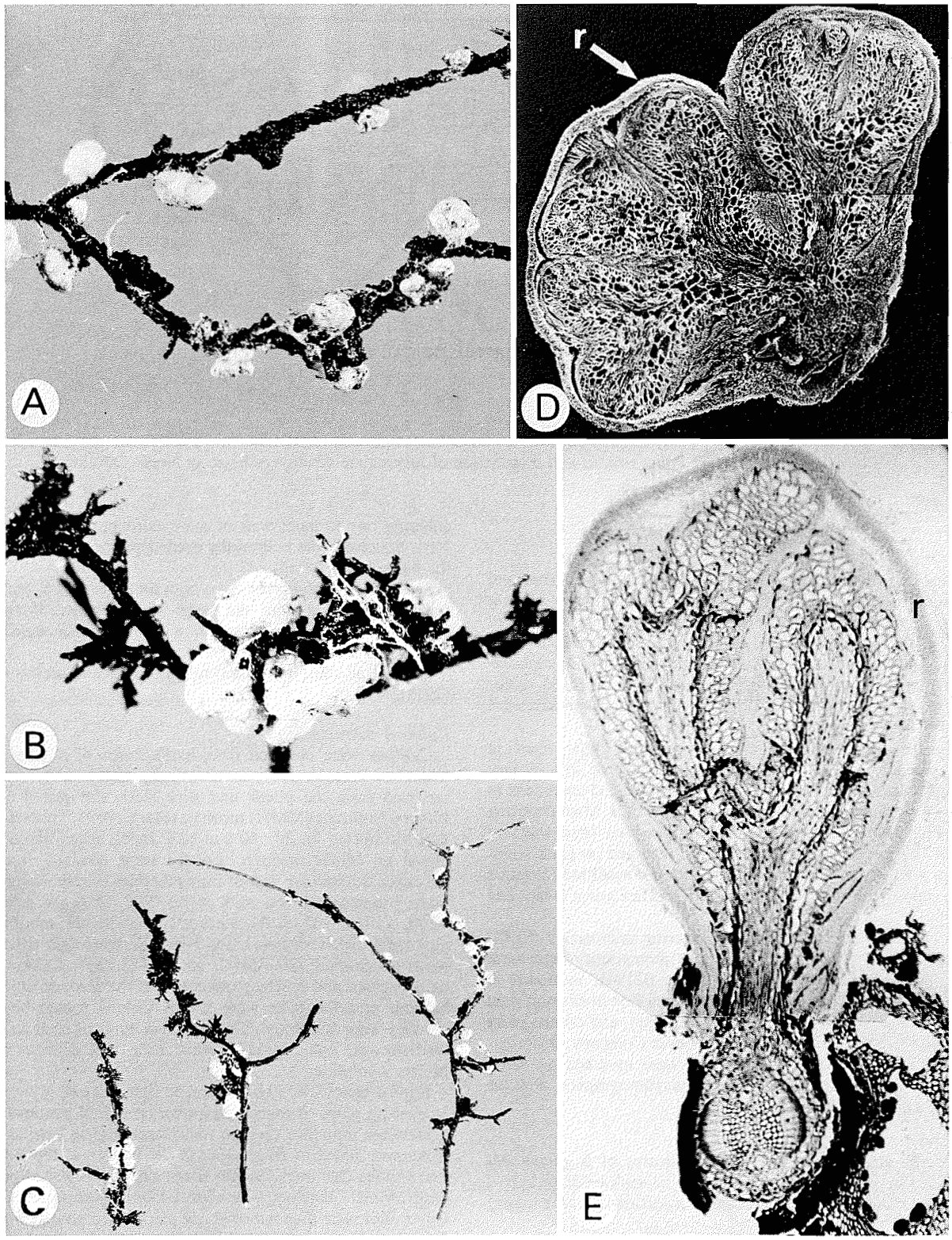

FIG. 2. Tuberculate ectomycorrhizae of *Pinus strobus*. Figs. 2A–2C. Gross morphology. Fig. 2A. ×5. Fig. 2B. ×3.5. Fig. 2C. ×1. Fig. 2D. SEM transverse section. ×25. Fig. 2E. Light microscopy cross section. ×11.7. *r*, rind.

4-day intervals for 28 days. The test was repeated at each of the six temperatures. Isolates T50, T100, T102, and T104 were also grown at 8°C.

Results

Tubercules

Tubercles were found at all mountain populations examined (Avery, Watauga, Buncombe, Macon, Henderson, and Jackson counties), at a disjunct Piedmont population (Chatham County), and at two Piedmont plantations (Durham and Person counties) in North Carolina (Fig. 1).

Macroscopic characteristics

Tubercles from all locations exhibited a similar macroscopic

morphology (Figs. 2A, 2B, 2C). Tubercles varied in shape (evenly to irregularly globose to obovoid) and in size (0.6–6.0 × 0.4–9.0; average 2.1 × 2.4 mm). Color ranged from Pale Ochraceous Buff to Light Ochraceous Salmon to Pale Yellow Orange. Monopodial to more typically coralloid ectomycorrhizae (Figs. 2D, 2E) were encased in a mantle (henceforth the rind) with felty exterior mycelium, imparting a wooly appearance when viewed with a dissecting microscope. The rind was usually confluent with the mantle of the elements (henceforth the element mantle) at the tips and at the sides of marginal elements. Tubercles contained an average of 24 element tips.

Rhizomorphs emanated from the base of the tubercles and were similar in color to the tubercles. No other types of mycelial strands or hyphae were observed.

Microscopic characteristics

A prosenchymatous rind was continuous from the surface to the element mantle, with both rind and element mantle consisting of sheets of appressed, parallel hyphae (Fig. 3C). The hyphae were similar throughout the rind and element mantles, although differential staining occurred on the outermost hyphae of the rind with either safranin – fast green or modified Conant's Triarch (Fig. 3B). Outside the surface of the differentially stained hyphae was a layer of loose, felty mycelium. The hyphae of this mycelium were the same diameter as the mantle hyphae and stained similarly to the outermost sheets of rind hyphae. Clamp connections were present on rind and element mantle hyphae. Spaces or gaps occasionally occurred among elements and between elements and the rind. White to Light Pinkish Cinnamon to Tawny Olive hyphae or hyphal strands or sheets connected element mantles or element mantles and rinds. The composite mantle (rind plus element mantle) ranged in thickness from 43.2 to 129.5 with an average of 86.1 μm. The felty mycelium increased the mantle thickness by as much as 36.8 μm. The Hartig net (Fig. 3A, B, D) was well developed, penetrating three or four layers of cortical cells and was composed of one to several layers of hyphae, having more layers at the outer layer of cortical cells. Hyphal cells between the cortical cells were arranged in a labyrinthine pattern but appeared beaded in transverse section. The tannin layer was usually one cell thick, although it was discontinuous or absent in some samples.

Rhizomorphs

Histological examination of rhizomorphs from tubercles and from the base of *S. pictus* basidiocarps indicated anatomical similarities (Fig. 4).

Macroscopic characteristics

Rhizomorphs from both basidiocarps and tubercles ranged in color from Pale Ochraceous Buff to Light Ochraceous Salmon to Pale Yellow Orange. The surface was wooly, and the diameter varied from 0.06 to 0.30 mm (average diameter 0.24 mm), as rhizomorphs branched.

Microscopic characteristics

A pseudoparenchymatous shell of one or two cell layers (Fig. 4) was filled with small proscenchymatous cells (4.0 μm average diameter) and enlarged cells (26.7 μm average diameter). The enlarged cells had thickened walls (1.1–1.5 μm) compared with very thin walls (0.3–0.6 μm) of the small prosenchymatous cells. Pores apparently connected enlarged cells in a longitudinal plane (Fig. 4A). Loosely interwoven hyphae (1.5–3.0 μm) sometimes encompassed the pseudoparenchymatous shell. The cells of the pseudoparenchymatous shell had an average dimension of 14.5 × 8.0 μm.

Chemical reagent color reactions

Tubercles and rhizomorphs

The most reactive tissues were the rhizomorphs and the rind surface of the tubercles. Little or no reaction was observed with most of the chemical reagents on internal hyphae of the rind, element mantle, or pine tissues.

The rind surface of fresh tubercles turned Corinthian Pink in fumes of NH_3 changing to Scarlet or Orange Buff when moistened with H_2O. Exposure of the rind surface to NH_4OH solution resulted in an immediate color change from buff to hues of red and pink and finally to deep lavenders. No color reactions were observed on the inside of the rind or on the pine tissue after exposure to NH_3 fumes or NH_4OH. Reactions similar to those on the rind were noted on rhizomorphs of both *S. pictus* and tubercles.

Rind surfaces immediately turned Rose Red and slowly darkened to purple in 10% aqueous KOH and became drab purple after 10 min. Rhizomorphs became pink, then transparent, and appeared to expand. Rhizomorphs attained a purplish hue within a few minutes.

Concentrated H_2SO_4 immediately darkened and slowly disintegrated tubercle sections. Sections removed from the solution were Russett or Mars Brown. Rhizomorphs from tubercles exhibited the same reactions.

Rhizomorphs displayed identical color reactions whether they emanated from the base of *S. pictus* basidiocarps or from tubercles.

Basidiocarps of Suillus pictus

NH_4OH and fumes of NH_3 turned the base of the stipe and the stipe context initially red then vinaceous, even after the tissue was dried. The colors, however, faded in 15–20 min. Color reactions similar to those previously reported for this fungus occurred on pileus and stipe flesh, pores, and the base of the stipe (Grund and Harrison 1976; Harrison and Grund 1975; Singer 1945).

Cultural characteristics of isolates from Suillus pictus *and tuberculate ectomycorrhizae*

Cultural characteristics and chemical reactions did not differ significantly between *S. pictus* and tubercle isolates. Details of these features are described and any notable differences between *S. pictus* and tubercle isolates are indicated.

Macroscopic characteristics

Mycelium aerial, appressed, with some submerged hyphae. Initial, freshly isolated cultures on MMN medium White to Pale Buff to Pallid Mouse Gray; considerable variation with isolate, medium, and age in color and relief of hyphae (Table 1). Reverse color slightly to strongly zonate. Reverse zonation on PDA slight with central areas Mars Brown to Verona Brown and marginal areas Honey Yellow to Cinnamon Buff, Cinnamon Brown, Van Dyke Brown, to hyaline. Reverse zonation on MMN medium more pronounced with central areas of Sepia to Raw Umber and marginal areas of Tawny Olive, Saccardo's Umber, Tawny, or Cinnamon Brown. Reverse zonation on MH medium moderate to strongly pronounced with considerable variation in color of central areas (Pale Olive Buff, Clove Brown, Hare Brown, Aniline Black, Deep Stately Brown) and marginal areas (usually Saccardo's Umber or Snuff Brown). Diffusable pigment intense on PDA, moderately to slightly intense on MMN, and not at all on MH

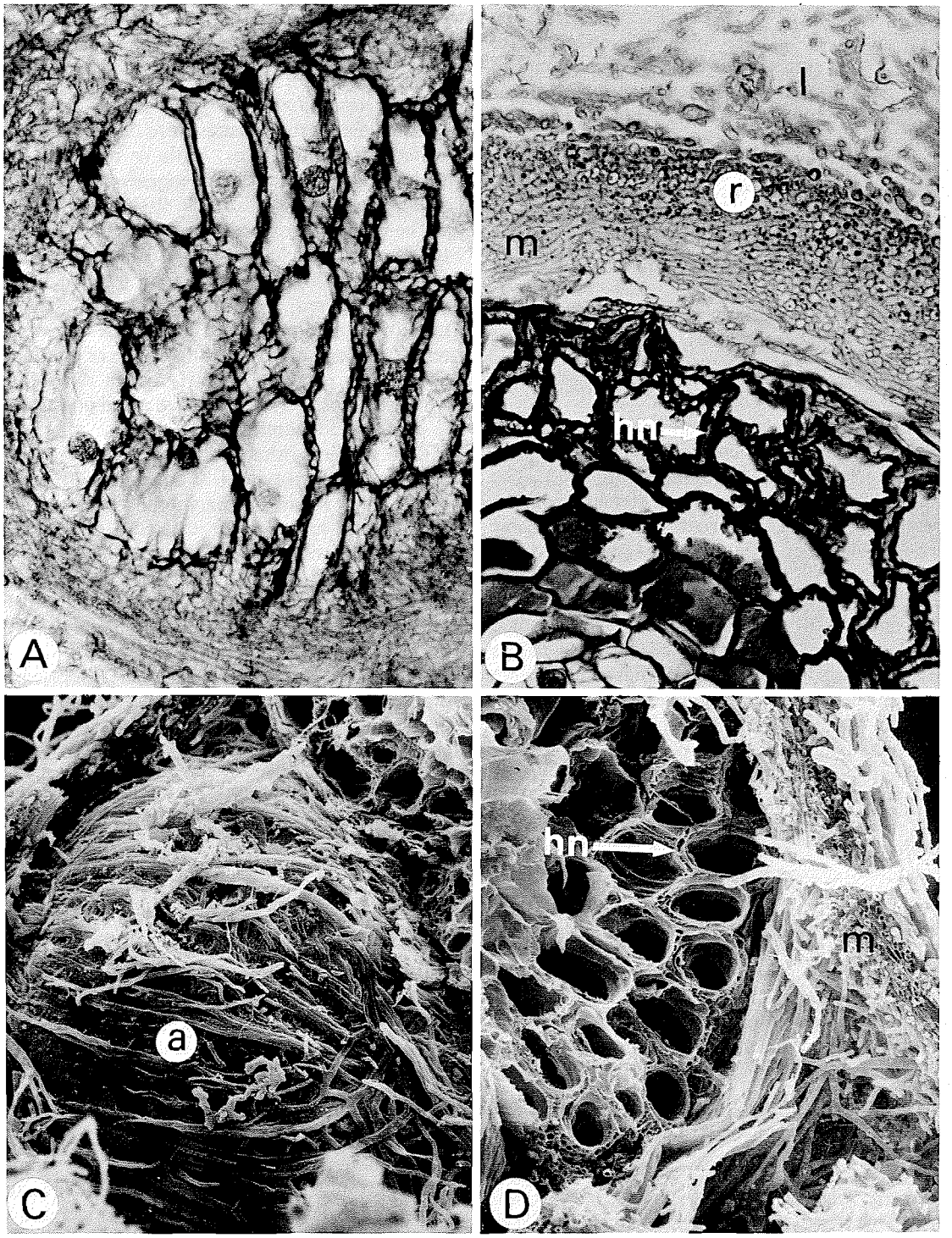

FIG. 3. Mantle and Hartig net of tuberculate ectomycorrhizae of *Pinus strobus*. Fig. 3A. Transverse section of cortical cells and mantle. ×320. Fig. 3B. Cross section of element. ×320. Fig. 3C. SEM of appressed nature of mantle hyphae (*a*). ×270. Fig. 3D. SEM of cross section of element. ×270. *l*, loose mycelium; *hn*, Hartig net; *m*, mantle; *r*, rind; *a*, appressed hyphae.

media. Pigment on PDA medium at room temperature Mustard Yellow to Primuline Yellow becoming darker with increasing colony diameter and (or) age to Cinnamon Brown near the mycelium and Cinnamon Buff at the edge of the Petri plate; pigment on MMN medium at room temperature none to pale or faint Naples Yellow or Mustard Yellow.

Colony topography and morphology generally consistent for all isolates on a particular medium. Central area on PDA medium appressed fibrillose; margin appressed fibrillose, even, with wooly to cottony aerial rings dividing central area from margin; number of rings variable, usually one or two. Central area on MMN medium, wooly to appressed; margin wooly if one or two cottony aerial rings present between center and margin; if no rings present, margin cottony. Margin even, becoming irregular with age. Mycelium on MH medium wooly and aerial with a narrow, slightly appressed margin.

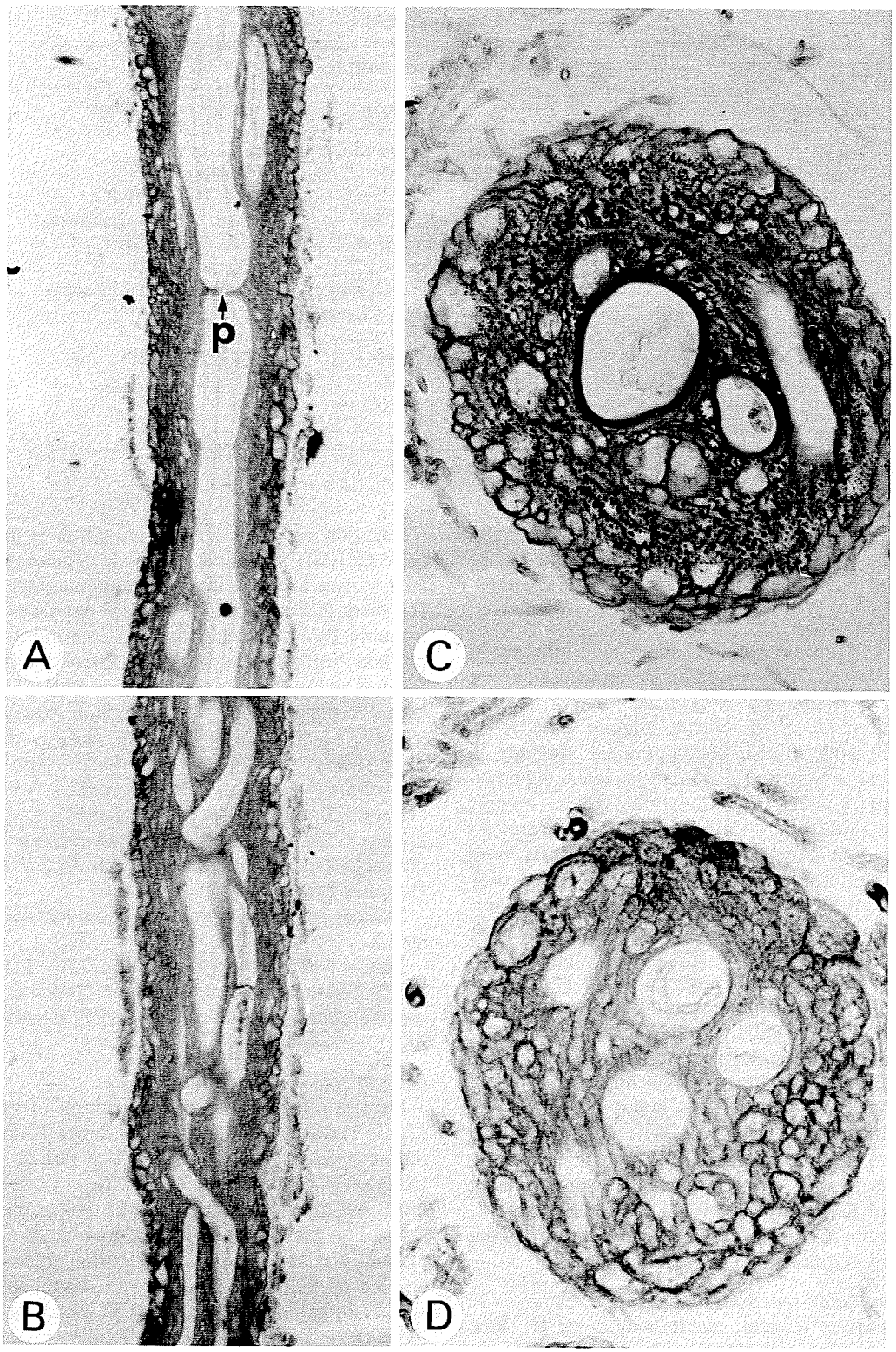

FIG. 4. Light microscopy of rhizomorphs from *Suillus pictus* and from tuberculate ectomycorrhizae of *Pinus strobus*. Figs. 4A and 4C. Rhizomorphs of *S. pictus*. Fig. 4A. Longitudinal section. ×150. Fig. 4C. Cross section. ×340. Figs. 4B and 4D. Rhizomorphs of tuberculate ectomycorrhizae. Fig. 4B. Longitudinal section. ×150. Fig. 4D. Cross section. ×670. Note Fig. 4A, pores (*p*) between vessel-like cells in longitudinal section of rhizomorph.

Sectoring noted in only one tuberculate isolate (T104) on both MMN and MH media. Lateral folding noted only in two tuberculate isolates, but each isolate exhibited the characteristic on different media: on PDA for T101 and on MH for T104.

No droplets of liquid or rhizomorphs observed. Hyphal strands occasionally in the central zones of older cultures of

TABLE 1. Range of colors observed for mycelium of *Suillus pictus* and tubercle isolates from *Pinus strobus* on three media

Medium	Cultural position		
	Center	Margin	Aerial rings
PDA	Light Pinkish Cinnamon*	Pale Brownish Drab*	Pinkish Buff*
	Cartridge Buff	Cartridge Buff	Cartridge Buff
	Vinaceous Cinnamon	Tilluel Buff	Pale Pinkish Buff
	Pinkish Buff	Pale Pinkish Buff	Light Pinkish Cinnamon
MMN	Pale Pinkish Buff to	Pale Pinkish Buff*	Pale Pinkish Buff
	Pinkish Buff*	Pinkish Buff	Cartridge Buff
	White	Pale Pinkish Cinnamon	Light Pinkish Cinnamon
	Pale Pinkish Cinnamon	Light Pinkish Cinnamon	
	Cartridge Buff		
MH	Cartridge Buff*	Cartridge Buff*	Cartridge Buff*
	Pale Pinkish Buff	Buffy Brown*	
	Pale Pinkish Cinnamon	Ivory Yellow	
	Ivory Yellow		

*Colors most frequently observed. Colors are from Ridgeway (1912).

S. pictus; although strand initials formed on PDA medium by only one tuberculate isolate (T104). A faint pleasant odor often discernible on PDA and MMN; no discernible odor on MH.

Microscopic characteristics

Hyphae (1.5–7.5 μm) smooth or incrusted, straight to undulate or helicoid. Undulate or helicoid hyphae generally smaller in diameter (1.5–4.5 μm) than straight hyphae (1.5–7.5 μm). Hyphae of *S. pictus* slightly smaller in diameter (1.5–7.0 μm). Bluish black granular contents in some smooth-walled hyphae in tuberculate isolates; spherical to elongate granular contents common in *S. pictus* isolates. Intercalary hyphal swellings occasionally in small-diameter incrusted hyphae. Hyphal swellings at the point of branching in both incrusted and smooth-walled hyphae; however, only slight swellings on smooth-walled hyphae of average diameter. Rarely a hypha coiled into a single loop in tuberculate isolates. Clamp connections produced at 1–8% of the septations on main hyphae in tuberculate isolates and at 1–10% in *S. pictus* isolates; frequency varied among isolates. Anastomosing hyphae observed rarely. Hyphal strands common, usually three to six hyphae per strand. Hyphal strands were occasionally absent in tuberculate cultures. Four hyphal branching habits were observed and percentage is based on presence at septations (Grand 1968): type 1, a single branch formed behind a septum on the main hyphae, 30%; type 2, paarige branching, 3%; type 4, paarige branching with a branch arising from one of the paarige branches, 2.5%; and type 8, two branches from the main hyphae, one from a clamp and the other from the main hyphae opposite the clamp, 3%.

Chemical reagent color reactions

NH_4OH most reactive reagent; results similar on all three media. Mycelium on PDA and MMN media beneath and next to drop of NH_4OH Light Plumbago Gray to Rood's Lavender; farther from the drop appressed hyphae responded to volatized fumes from the drop turning Livid Pink to Corinthian Pink; appressed hyphae (margin) turning Rood's Lavender to Pale Purple Drab; entire mycelial mat some shade of purple to pink within 10 min, and within 1 h aerial hyphae Orange Vinaceous to Pale Purple Drab. Reactions on MH similar but colors more faint, fading within 5 min; within 1 h entire mycelial mat, Pallid Vinaceous Drab to Pallid Quaker Drab.

Reaction to KOH similar on all three media. Mycelium beneath KOH drop instantly Pale Vinaceous on PDA, then Pale Vinaceous Drab in central and marginal (appressed) areas and Dark Purple Drab on the aerial hyphae; on MMN medium instantly Pale Vinaceous and within 3 min Dull Indian Purple or Slate Purple. Areas beyond drop eventually Vinaceous Gray (central and marginal areas) or Vinaceous Gray to Purple Drab (aerial hyphae); drop on MH medium slowly Vinaceous Gray in tuberculate isolates; *S. pictus* isolates responding a little more slowly from a Vinaceous shade to finally a pale or faint Vinaceous Gray. Margin a more intense Vinaceous Gray.

No reaction of mycelium to Melzer's reagent on all media; however, the PDA medium turned immediately Dark Amber Green to Dusky Blue Green, then Dusky Blue, and finally Hortense Blue.

Sulfuric acid negative on mycelium and medium for all three media.

Tuberculate cultures used were T50, T100, T101, T102, T104 (Chatham County), and T3 (Jackson County). *Suillus pictus* cultures used were SP1, SP7 (Chatham County), and SP5 (Jackson County).

Growth rate of tuberculate isolates

Diameter growth on MMN medium of tuberculae isolates (Table 2) was optimal at 24 (49.8 mm at 1 month) and substantial at 20 and 16°C (44.5 and 34.5 mm at 1 month, respectively). Growth occurred at both 8 (2.3 mm) and 12°C (11.4 mm), but rates were much slower than at the higher temperatures. The growth rate was moderate at 28 (23.8 mm) and no growth was observed at 32°C. Mat coloration and relative amount of diffusable pigment varied among isolates and with temperatures. Isolates T3 and T50 produced a diffusable pigment at 24 and 28°C. Isolate T3 also produced a diffusable pigment at 30°C. Little or no pigment was produced by these isolates at temperatures below 24°C. Isolates T102 and T104 produced a pigment, but coloration was less intense. T100 produced no observable pigment. Isolates T100, T102, and T104 all displayed some degree of sectoring.

Discussion

Proven mycobionts of tuberculate ectomycorrhizae have been species of *Rhizopogon* (Trappe 1965; Zak 1971) and

TABLE 2. Diameter growth rate (mm) after 1 month of five tuberculate isolates from *Pinus strobus* L. in North Carolina on modified Melin–Norkrans medium at seven temperatures*

Isolate	Temperature (°C)†					
	8	12	16	20	24	28
T3	—	14.6	46.0	56.7	61.7	30.6
T50	1.4	5.7	15.7	30.8	38.7	18.0
T100	2.8	12.4	32.1	44.2	49.7	24.6
T102	2.3	10.9	23.6	41.6	48.8	21.0
T104	2.9	12.8	41.0	49.3	50.0	25.0
Average	2.3	11.4	34.5	44.5	49.8	23.8

*Average of 10 plates per isolate per temperature. There was no difference between two growth studies, hence data were pooled.
†No growth occurred at 32°C.

Suillus (Chumak 1981; Melin 1922, 1923, 1924). Smith (1971) related these species in a hypothetical evolutionary series: *Rhizopogon–Truncocolumella–Suillus–Fuscoboletinus–Gomphidius–Chroogomphus*. All of these genera include species which form ectomycorrhizae with conifers. Members of the Gomphidiaceae (*Chroogomphus rutilus* (Schaeff : Fr.) O. K. Miller, *Gomphidius maculatus* (Scop. : Fr.) Fr.) and *Rhizopogon* (*R. nigrescens* Coker & Couch, *R. rubescens* (Tul.) Tul., *R. subaustralis* A. H. Sm.) were collected from the disjunct population study site (B. L. Randall and L. F. Grand, unpublished data). The most commonly represented genus of this series, however, was *Suillus* (*S. americanus, S. granulatus,* and *S. pictus*). Of these species, *S. pictus* was the most frequently associated with *P. strobus* tubercles (B. L. Randall, unpublished data).

Macroscopic chemical color reactions on tubercles, rhizomorphs from tubercles, and cultures of tubercle isolates were very similar to those color reactions on the base of the stipe, the rhizomorphs, and cultures of *S. pictus*. Macroscopic and microscopic cultural characteristics observed for isolates of *S. pictus* and of the *P. strobus* tubercle were also very similar.

Pantidou (1961) observed a smaller range of hyphal diameter for *S. pictus* than was observed in this study. The smooth mycelial sheet observed by Pantidou was not obvious, but hyphal strands were observed. Clamps were seen on the branches, though rarely. A diffusable pigment was produced by cultures of *S. pictus* and tubercule isolates on both PDA and MMN media (intensity was temperature dependent on MMN medium) but were not observed by Pantidou (1961). One or more cultural characteristics (macroscopic and microscopic features, growth rates, and chemical reactions) of the tubercle isolates differed from those reported by Pantidou (1966) for *S. albidipes, S. americanus, S. granulatus,* and *S. punctipes.*

A similar range of macroscopic chemical color reactions was observed on all *P. strobus* tubercles examined. Chemical color reaction data are not available in the literature from tubercles described by Melin (1921, 1922) and Chumak (1981). Zak (1971) reported chemical color reactions for *Rhizopogon vinicolor + Pseudotsuga menziesii*. Although responsive to reagents, such as NH_4OH and KOH, the reactions were not like those observed with *P. strobus* tubercles. No color reactions were observed by Zak for Melzer's reagent and concentrated sulfuric acid. The most reactive tissue as *P. strobus* tubercles was the rind.

All tubercles examined from *P. strobus* plantations and natural populations exhibited the same basic morphology and were similar to those reported on other *Pinus* spp. (Melin 1921, 1922, 1923, 1924; Chumak 1981).

Only a few *Rhizopogon* species have been found in association with *P. strobus* in North Carolina. None observed have buff-colored rhizomorphs. The rhizomorphs from tubercles and from *S. pictus* are anatomically identical. These structures are similar to those described by Melin (1923) from "Knollenmykorrhiza" on *Suillus luteus + Pinus sylvestris* and on *Suillus* sp. + *P. sylvestris*. Differences between Melin's description and observations presented here for tubercles on *P. strobus* are cell diameters and cell differentiation. Three distinct zones plus a felty covering were present in tubercle and *S. pictus* rhizomorphs. Only two zones were noted by Melin (1923). Similarities between observations reported here and by Melin include the large vessel-like cells in the center of the tubercles, the pseudoparenchymatous shell, and the felty covering. The zone of prosenchymatous cells between the large vessel-like cells and the pseudoparenchymatous shell was not noted by Melin. Chumak (1981) did not mention the prosenchymatous layer in the rhizomorph. Duddridge *et al.* (1980) examined the ultrastructure of *Suillus bovinus* rhizomorphs produced in an unsterile peat medium with *Pinus sylvestris*. The anatomy was similar to *S. pictus* and tubercle rhizomorphs, but the middle zone of prosenchymatous cells was absent. The range in cell diameter for the vessel-like cells (6–20 μm) of *S. bovinus* rhizomorphs was close to that observed here with *S. pictus*. *Suillus bovinus* has not been reported from the United States.

Although certain cell characteristics differ from those of the *P. strobus* tubercle, a similar pattern of tissue differentiation was noted in *R. vinicolor + P. menziesii* by Zak (1971). The pseudoparenchymatous sheath of *R. vinicolor + P. menziesii* was thicker than the tubercles in this study.

As with the anatomical similarities of *S. pictus* and tubercle rhizomorphs, chemical color reactions with these structures yielded similar results. These results were the same as those observed on the outer layer of the tubercle mantle and on the base of basidiocarps of *S. pictus*. Reactions observed on *S. pictus* pileal context with $FeSO_4$, KOH, and NH_4OH were the same as those described by Grund and Harrison (1976), Harrison and Grund (1975), and Singer (1945). The "fleeting pink" initial response to KOH reported by Baroni (1978) was not observed.

Pure culture synthesis of *S. pictus* (= *Boletinus pictus*) + *P. strobus* ectomycorrhizae has been reported by Doak (1934) and Hatch (1936). No descriptions or pictures were given by Doak or Hatch for *S. pictus + P. strobus* ectomycorrhizae. Doak did describe and illustrate the pure culture synthesis of *S. pictus + Pinus rigida* Mill. ectomycorrhizae. The element illustrated was similar to a *P. strobus* tubercle having a thick mantle, a discontinuous tannin (epidermal) layer, and a Hartig net. The Hartig net penetrated to the endodermis and was several cells thick at the tannin and outermost cortical cell layers. No mantle rind or rhizomorphs were observed, although sparse mycelial growth occurred in the sand (potting medium).

Palm and Stewart (1984) examined several *Suillus–Pinus* associations *in vitro*. The *S. pictus + P. strobus* ectomycorrhizae had several features in common with the tuberculate ectomycorrhizae. The mantle thickness, felt parenchyma on mantle surface, and basic form of entire ectomycorrhizae are similar to the tubercle. Some of the *in vitro* ectomycorrhizae were "quite swollen" and "covered with a dense 'orange'

sheath." Although not described as a tuberculate form, a sheath over a short, coralloid to palmate clustered ectomycorrhiza would closely resemble a coralloid ectomycorrhiza encased in a mantle rind. Colors of the *S. pictus* + *P. strobus* ectomycorrhizae and associated hyphal strands described by Palm and Stewart are more intensely orange than any tubercles encountered by the authors. However, the mantle of the tubercle is chemically reactive as noted in this study and may be responsive to yet other chemicals by a change in mantle coloration. A major difference between the described *S. pictus* + *P. strobus* ectomycorrhizae reported by Palm and Stewart and the tubercule in this study is in the orientation of the hyphae within the mantle. Definite sheets of parallel hyphae occur in the mantle of the tubercle but were not described by Palm and Stewart. Some "bands" of parallel hyphae were observed by Palm and Stewart when *Suillus brevipes* was the mycobiont. The labyrinthine nature of the Hartig net and the innermost layers of mantle in the tubercle are more regular and interlocking than the illustration of irregular synenchyma illustrated for *S. pictus* + *P. strobus* ectomycorrhizae. Palm and Stewart used a single isolate of *S. pictus*.

Preliminary pure culture synthesis of a *S. pictus* + *P. strobus* ectomycorrhizae yielded a thick mantle overlapping between elements but without a definite rind or even edge to the mantle (B. L. Randall, unpublished). This ectomycorrhiza greatly resembled the *S. pictus* + *P. rigida* ectomycorrhiza synthesized by Doak (1955). Thus, with varying techniques and host sources and different mycobiont isolates, some variation occurred among synthesized *S. pictus* + *P. strobus* ectomycorrhiza.

Further study is needed to determine the variation among mycobiont capabilities of *S. pictus* isolates and conditions involved in the synthesis. Duddridge *et al.* (1980) exposed *Suillus bovinus* + *Pinus sylvestris* synthesis to an unsterile potting medium to induce rhizomorph formation. Certain conditions or factors may be needed to form a complete tubercle and rhizomorphs in the *S. pictus* + *P. strobus* association.

Strong circumstantial evidence is presented to support the hypothesis that the tubercle on *P. strobus* is formed by association with *Suillus pictus*: (*i*) the anatomical similarities of tubercle and *S. pictus* rhizomorphs; (*ii*) similar chemical color reactions with rhizomorphs of tubercles and *S. pictus*, with tubercle mantles, and with the base of the stipe of *S. pictus* basidiocarps; (*iii*) similar chemical color reaction with cultures of *S. pictus* and the tubercle mycobiont; (*iv*) similar hyphal characteristics and mat growth of isolates of *S. pictus* and the tubercle mycobiont; and (*v*) the consistent field association between *S. pictus* and tubercles.

Acknowledgements

This research was supported in part by a grant-in-aid from The Highlands Biological Station, Highlands, North Carolina. We gratefully acknowledge Dr. Gerald Van Dyke for the scanning electron microscopy. Access to the disjunct population was graciously provided by Mr. and Mrs. Paul Kamp. Photographic technical assistance was provided by Mr. Marvin Williams and Mr. William E. Schadel.

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